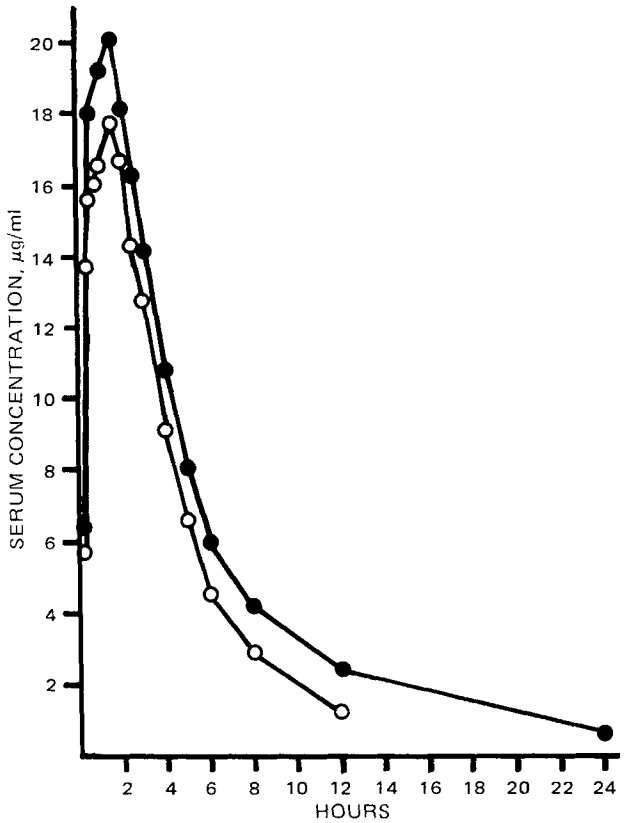

Figure 2—*Serum I concentration after oral administration of 200 mg of I in an aqueous medium. Key (mean values of eight subjects): ●, ^{14}C-assay; and ○, UV spectrophotometric assay.*

study indicated good day-to-day reproducibility on samples assayed over 3 weeks.

The composite linear regression standard curve had a y-intercept of 0.024 absorbance unit (blank), a slope of 0.018 absorbance unit/μg of I/ml, and $r^2 = 0.995$ ($y = 0.018x + 0.024$), where x is micrograms of I per milliliter of serum. The coefficient of variation was 5% with serum samples containing 2, 5, 10, 20, and 30 μg of I/ml on 12–14 determinations of each concentration over 3 weeks.

Replicate determinations of I in 10 identical serum standards, each containing 20 μg of I/ml, on the same day resulted in a coefficient of variation of 2.8% and a mean of 0.321 ± 0.009 absorbance unit at 252 nm.

Sensitivity—As mentioned previously, linear regression analysis of absorbance *versus* standard concentration data yielded a slope of 0.018 absorbance unit for 1 μg of I/ml of serum. When using 1.5 ml of serum per test, the minimum detectable quantity of I was approximately 1 μg/ml.

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Quantitation of the Antimalarial Agent, Mefloquine, in Blood, Plasma, and Urine Using High-Pressure Liquid Chromatography

J. M. GRINDEL *ˣ, P. F. TILTON *, and R. D. SHAFFER ‡

Abstract □ Sensitive and specific assays are described for the quantitation of mefloquine in whole blood, plasma, and urine specimens using high-pressure liquid chromatography. Specimens were extracted with ethyl acetate and concentrated before chromatography. Whole blood and plasma extracts were chromatographed on a polar bonded phase partitioning column, and urine extracts were chromatographed on a bonded reversed-phase partitioning column. The sensitivity of the assays for mefloquine was 0.05 μg/ml of whole blood or plasma and 0.25 μg/ml of urine using 5-ml samples. The assays are suitable for studying mefloquine pharmacokinetics in humans.

Keyphrases □ Mefloquine—high-pressure liquid chromatographic analysis, whole blood, plasma, and urine □ High-pressure liquid chromatography—analysis, mefloquine in whole blood, plasma, and urine □ Antimalarial agents—mefloquine, high-pressure liquid chromatographic analysis in whole blood, plasma, and urine

Mefloquine hydrochloride[1] (I) is a radical curative agent for the treatment of drug-resistant falciparum malaria (1, 2). Mefloquine also provided suppressive prophylaxis against mosquito-induced infections with *Plasmodium vivax* and *P. falciparum* in human volunteers (3, 4). The disposition of mefloquine in rats was studied (5), but its fate in humans has not been determined due to the lack of

[1] DL-*erythro*-α-2-Piperidyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride (WR 142,490 hydrochloride), Cordova Chemical, Sacramento, Calif.

Table I—Retention Volumes (V_R) for I and II on Reversed-Phase or Normal Phase Partition Chromatography

Mobile Phase	V_R, ml I	V_R, ml II
Bonded Reversed-Phase Partition Chromatography[a]		
Methanol–0.1 *M* NaH_2PO_4		
3:2 (v/v)	5.4	4.0
2:1 (v/v)	4.6	2.9
4:1 (v/v)	3.0	2.2
Polar Bonded Phase Partition Chromatography[b]		
Isopropyl ether–dioxane–acetic acid		
3:2 (v/v) + 0.5%	4.4	10.3
2:3 (v/v) + 0.5%	3.7	6.5
1:4 (v/v) + 0.5%	20.5	42.5

[a] μBondapak C_{18} column (4 mm i.d. × 30 cm). [b] μBondapak CN column (4 mm i.d. × 30 cm).

a sensitive and specific nonradioactive assay.

High-pressure liquid chromatography (HPLC) is a powerful analytical technique for the quantitative analysis of pharmaceuticals in biological fluids. Due to the low vapor pressure of mefloquine, GLC was not as applicable as HPLC. Thus, HPLC was utilized to effect a specific separation and quantitation of mefloquine from whole blood, plasma, and urine specimen extracts.

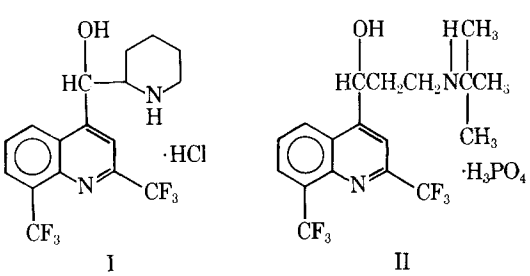
EXPERIMENTAL

Reagents—Mefloquine hydrochloride and the internal standard[2] (II) were assayed at >99 and 98% purity, respectively. All solvents used were spectroquality, and drug solutions were made with the appropriate salt form of the compound. All concentrations are expressed as the free base.

Standard Solutions—A solution of the internal standard was prepared in dioxane–methanol (7:3 v/v) (196 μg/ml). The internal standard solution was used as the solvent for the preparation of a stock solution of mefloquine (1028 μg/ml), from which dilutions were made with the internal standard solution to give solutions with concentrations of 10.2–205.6 μg of mefloquine/ml and 1.96 μg of II/ml.

Chromatographic Conditions—A high-pressure liquid chromatograph[3] equipped with a 280-nm absorbance monitor was utilized. Whole blood and plasma extracts were chromatographed on a column of 10-μm, fully porous silica, bonded with a monomolecular layer of cyanopropylsilane[4]. A mobile phase of isopropyl ether–dioxane–acetic acid (3:2 v/v + 0.5%) at 2 ml/min was used. Urine extracts were chromatographed on a column of 10-μm, fully porous silica, bonded with a monomolecular layer of octadecylsilane[5]. A mobile phase of methanol–0.1 *M* NaH_2PO_4 (3:2 v/v) at 2 ml/min was used.

General Assay Procedure—The internal standard solution (50 μl) containing 9.8 μg of II, 5 ml of whole blood, plasma, or urine, and 5 ml of pH 7.4 phosphate buffer (0.065 *M*) were placed in a 45-ml glass conical tube with a polytef[6]-lined screw cap and thoroughly mixed. Then 10 ml of ethyl acetate was added to each tube, and the samples were agitated at 100 strokes/min for 30 min on a reciprocating shaker bath[7] and centrifuged[8] at 2000 rpm for 10 min. The ethyl acetate layer was then pipetted carefully into a 45-ml glass conical tube with a polytef stopper.



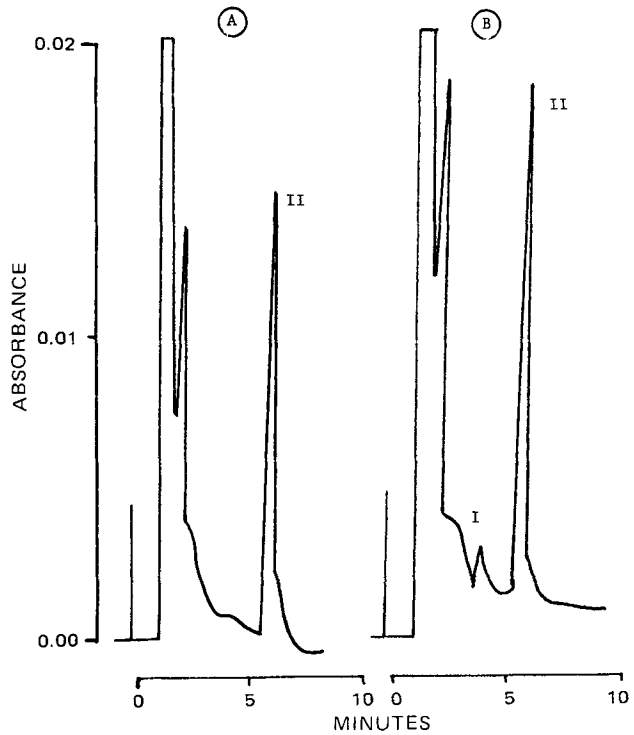


Figure 1—*Representative chromatograms of a whole blood extract containing 0.98 μg of II (A) and of a whole blood extract containing 0.05 μg of I plus 0.98 μg of II (B) after chromatography on a polar bonded phase column using isopropyl ether–dioxane–acetic acid (3:2 v/v +0.5%) as the mobile phase.*

The extraction procedure was repeated two more times, the combined ethyl acetate layers from each sample were evaporated to dryness under a gentle stream of nitrogen at 40° [9], and the residues were stored overnight in a vacuum desiccator. The sample residues were reconstituted with 500 μl of the appropriate mobile phase, and 50-μl aliquots were injected into the chromatograph. Quantitation was achieved by measuring peak area ratios for I/II and relating them to the least-squares linear regression curve of the peak area ratio for I/II *versus* the amount of mefloquine injected. Peak areas were obtained from the product of the maximum peak height (centimeters) × width at peak half-height (centimeters).

Spiked Samples—*Recovery of Mefloquine from Whole Blood*—Aliquots (25 ml) of human whole blood were spiked with a methanol solution of mefloquine (250 μg/ml) to give drug concentrations of 1.00, 0.50, and 0.10 μg/ml. The samples were thoroughly mixed for 30 min and treated as described under *General Assay Procedure*, except that the internal standard (II) was not added until the extraction was completed.

Recovery of II from Whole Blood—Aliquots (25 ml) of human whole blood were spiked with a methanol solution of II (250 μg/ml) to give drug concentrations of 1.00, 0.50, and 0.10 μg/ml. The spiked blood was thoroughly mixed for 30 min, and four 5-ml samples of each concentration level were placed in individual glass tubes along with 5 ml of pH 7.4 phosphate buffer (0.065 *M*). Each sample was extracted with 3 × 10 ml of ethyl acetate, the combined ethyl acetate extracts were evaporated under nitrogen, the residues were reconstituted with 500 μl of mobile phase, and 50-μl aliquots were injected into the chromatograph. Quan-

[2] DL-2,8-Bis(trifluoromethyl)-4-[1-hydroxy-3-(*N-tert*-butylamino)propyl]quinoline phosphate (WR 184,806 phosphate), Starks Associates, Buffalo, N.Y.

[3] A model 6000A solvent delivery system, a model U6K injector, and a model 440 UV absorbance monitor, Waters Associates, Milford, Mass.

[4] μBondapak CN column (4 mm i.d. × 30 cm), Waters Associates, Milford, Mass.

[5] μBondapak C_{18} column (4 mm i.d. × 30 cm), Waters Associates, Milford, Mass.

[6] Teflon, du Pont.

[7] Model 2156, American Optical Corp., Buffalo, N.Y. The tube holder assembly was fabricated by the Division of Instrumentation, Walter Reed Army Institute of Research, Washington, D.C.

[8] Model PR-2 refrigerated centrifuge, IEC, Needham Heights, Mass.

[9] Model 112 N-Evap, Organomation Associates, Northborough, Mass.

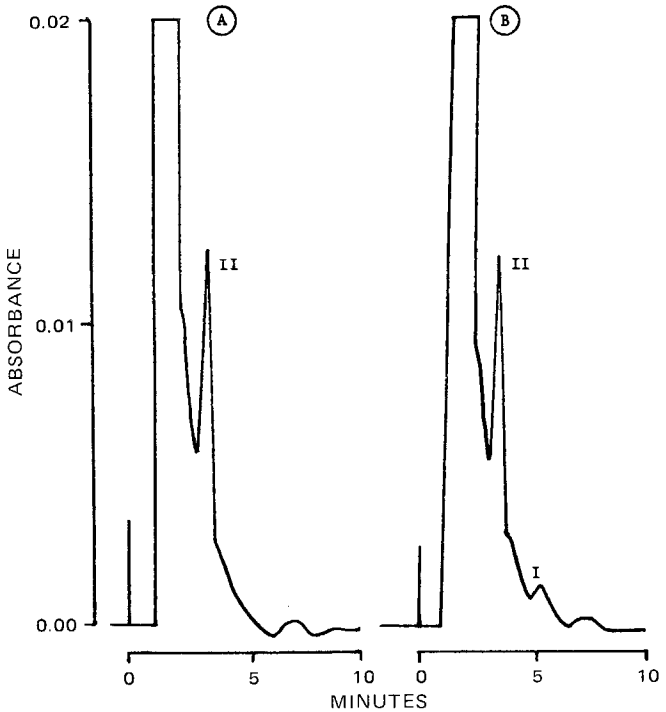


Figure 2—*Representative chromatograms of a urine extract containing 0.98 μg of II (A) and of a urine extract containing 0.125 μg of I plus 0.98 μg of II (B) after chromatography on a bonded reversed-phase column using methanol–0.1 M NaH_2PO_4 (3:2 v/v) as the mobile phase.*

titation was achieved by measuring the peak areas of II and relating them to the least-squares linear regression curve (n = 15) of the peak area of II *versus* the amount of II injected. Peak areas were measured as described previously.

Precision Determination for Mefloquine Assay—Aliquots (25 ml) of human whole blood and plasma were spiked with a methanol solution of mefloquine (250 μg/ml) to give drug concentrations of 5.00, 1.00, 0.50, and 0.05 μg/ml. Urine aliquots (25 ml) were spiked at 5.00, 1.00, 0.50, and 0.25 μg/ml. The samples were thoroughly mixed for 30 min and treated as described under *General Assay Procedure*.

Clinical Study—An adult male was given two 250-mg tablets of mefloquine hydrochloride. Whole blood and plasma samples were obtained at 0, 1, 2, 4, 8, and 12 hr and at 2, 3, 4, 7, 9, 14, 21, 31, and 57 days. A random urine sample was obtained on Days 1 and 2. All specimens were stored in glass tubes with polytef-lined screw caps at −10° until analyzed.

RESULTS AND DISCUSSION

Mefloquine and its analog II have similar physicochemical properties. Both compounds are extensively bound to plasma proteins and are concentrated in the erythrocytes (5, 6); they have nearly identical molar absorptivity at 280 nm, pKa's, solvent partition coefficients, and low aqueous solubility. Both compounds were well resolved from each other and from extracts of blood and urine under appropriate chromatographic conditions. For these reasons, II was chosen as the internal standard for the mefloquine assay.

The establishment of HPLC conditions was accomplished by examining the retention characteristics of mefloquine and II on polar bonded phase and bonded reversed-phase partitioning columns. Comparative retention volumes for mefloquine and II are presented in Table I. Optimal resolution of both compounds from each other and from ethyl acetate extracts of blood and plasma was accomplished using a polar bonded phase partitioning column with a mobile phase of isopropyl ether–dioxane–acetic acid (3:2 v/v + 0.5%). These conditions did not adequately resolve mefloquine and II from the urine extract peaks. Resolution of mefloquine and II from urine extracts was achieved on a bonded reversed-phase column utilizing a mobile phase of methanol–0.1 M NaH_2PO_4 (3:2 v/v). Representative chromatograms are presented in Figs. 1 and 2. No interfering peaks from blood, plasma, or urine extract components were detected in control samples.

Table II—Recovery of I and II from Spiked Whole Blood Specimens [a]

Mefloquine Added, μg/ml [b]	Recovery, % ± *SD*	
	I	II
1.00	95.4 ± 2.4	95.5 ± 1.3
0.50	99.1 ± 8.3	96.3 ± 4.9
0.10	105.2 ± 13.6	95.3 ± 9.3
Mean	99.9 ± 9.3	95.7 ± 5.4

[a] Chromatographic conditions are described under *Experimental*. [b] Four samples at each concentration level.

Table III—Precision Data for the Determination of Mefloquine in Spiked Whole Blood, Plasma, and Urine Specimens [a]

Mefloquine Added, μg/ml [b]	Assayed Mefloquine Concentration, μg/ml [c]		
	Whole Blood	Plasma	Urine
5.00	4.96 ± 0.25	5.20 ± 0.11	4.76 ± 0.14
1.00	1.04 ± 0.02	1.00 ± 0.06	0.97 ± 0.06
0.50	0.54 ± 0.04	0.47 ± 0.07	0.48 ± 0.05
0.25			0.25 ± 0.04
0.05	0.05 ± 0.004	0.05 ± 0.004	
Mean percent recovery ± *RSD*	102.9 ± 6.7	99.5 ± 9.5	97.1 ± 9.4

[a] Chromatographic conditions are described under *Experimental*. [b] The concentration of internal standard was 1.96 μg/ml. [c] Mean ± *SD* for four replicates at each level.

Recovery percentages for the individual extraction of mefloquine and II from whole blood using ethyl acetate as solvent are presented in Table II. The recoveries were greater than 95% for both compounds over the range studied with standard deviations less than 10%.

Linear calibration curves of the peak area ratio for I/II *versus* the amount of I were constructed each day utilizing 15 points at five concentration ratios. The data were analyzed using least-squares regression analysis. A family of curves over a 6-month period had a mean slope of 1.013 with a relative standard deviation of 3.9% and a correlation coefficient (r) of 0.9997.

Precision data utilizing optimal conditions for the assay of blood, plasma, and urine specimens spiked with known concentrations of mefloquine are presented in Table III. The relative accuracy was ±3% of the amount added with relative standard deviations less than 10% over the 0.05–5.00-μg/ml range for blood and plasma and over the 0.25–5.00-μg/ml range for urine. The lower limit of sensitivity for the assay was 0.05 μg/ml for blood and plasma samples and 0.25 μg/ml for urine samples.

Based on the results of these experiments, an adult male was given 500 mg of mefloquine hydrochloride, and serial blood and plasma specimens were assayed for 2 months for mefloquine concentrations (Figs. 3 and 4). Blood and plasma levels of mefloquine were readily quantitated 57 days after drug ingestion. Random urine specimens obtained on Days 1 and 2 were assayed for free mefloquine content (Table IV) and contained low, but quantifiable, levels of mefloquine.

The mefloquine blood and plasma level data from a single subject after oral administration indicated relatively rapid absorption, extensive distribution, and prolonged elimination phases. Partitioning of mefloquine into the erythrocytes in competition with extensive binding to

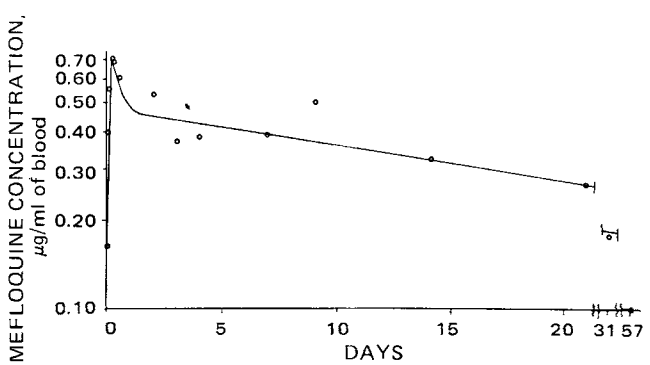


Figure 3—*Whole blood I concentrations in a male subject following a single oral dose of 500 mg of I.*

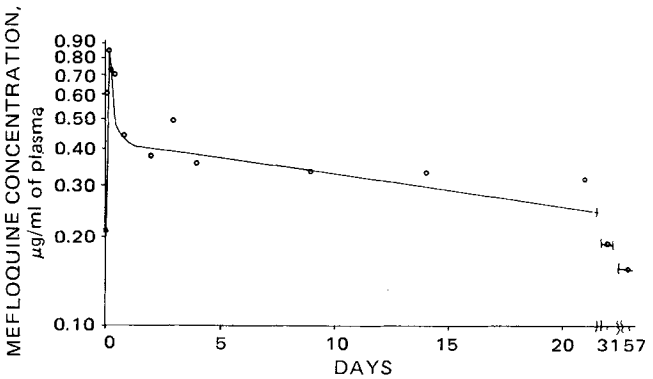


Figure 4—*Plasma I concentrations in a male subject following a single oral dose of 500 mg of I.*

plasma proteins probably caused the apparent disparities in the blood and plasma level data. Further data interpretation will be performed in conjunction with ongoing pharmacokinetic studies. The ability of the assay to follow blood and plasma levels of mefloquine for at least 57 days after a single oral administration of the drug established its utility for studying mefloquine's pharmacokinetic parameters in humans.

Table IV—Mefloquine Excretion in the Urine of an Adult Male after a Single Oral Dose of 500 mg of I [a]

	I Excretion		
Day[b]	μg/ml	μg/Sample	% Dose
1	3.03	527.92	0.12
2	0.76	127.01	0.03

[a] Chromatographic conditions are described under *Experimental.* [b] A random urine sample was obtained on each day.

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Cytolysis of Neuroblastoma Cells *In Vitro* and Treatment of Neuronal Tumors *In Vivo* with Bromoacetylcholine

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Abstract □ The effect of bromoacetylcholine on mouse neuroblastoma C-1300 was investigated in cell culture as well as in A/J mice. *In vitro,* bromoacetylcholine (1×10^{-5} *M*) was a potent cytolytic agent and produced an additive effect in combination with vincristine (3×10^{-9} *M*). Since the choline acetyltransferase inhibitor, dimethylaminoethyl chloroacetate, does not inhibit neuroblastoma efficiently *in vitro,* the potent cytolytic action of bromoacetylcholine is probably not due to its choline acetyltransferase inhibitory action. Furthermore, the neuroblastoma inhibitory effect of bromoacetylcholine was not affected by atropine. Therefore, the inhibitory action is not related to the interaction of bromoacetylcholine with muscarinic receptors either. In *in vivo* experiments, 1, 10, or 30 mg/kg of bromoacetylcholine was injected directly into the tumors three times daily for 6 weeks. Bromoacetylcholine at 10 and 30 mg/kg gave significant protection of A/J mice from the death induced by neuroblastoma inoculation, and the lifespan was prolonged significantly with these bromoacetylcholine treatments.

Keyphrases □ Bromoacetylcholine—*in vitro* cytotoxic and *in vivo* antitumor activity, mice □ Cytotoxic activity—bromoacetylcholine evaluated, mouse neuroblastoma cell culture □ Antitumor activity—bromoacetylcholine evaluated, mouse neuroblastoma □ Cholinergic agents—bromoacetylcholine, *in vitro* cytotoxic and *in vivo* antitumor activity, mice

Many attempts have been made to treat the highly malignant neuroblastoma in children (1). However, no drug tested, including cyclophosphamide and vincristine, is satisfactory, mainly because the remission rate in neuroblastoma patients is quite low and the duration of drug effectiveness is fairly short (2–4).

The nature of neuroblastoma cells can be cholinergic, adrenergic, or inactive (5–8). Acetylcholinesterase is always present, and these cells possess membranes that respond to acetylcholine and are excitable electrically. These data indicate the existence of cholinergic receptors at the membrane site (7, 9). Attempts were made to destroy these